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Urinary proteome pattern in children with renal Fanconi syndrome

Jens Drube[1,*], Eric Schiffer[2,*], Harald Mischak[2], Markus J. Kemper[3], Thomas Neuhaus[4], Lars Pape[1], Ralf Lichtinghagen[5] and Jochen H. H. Ehrich[1]

[1]Department of Paediatric Nephrology, Children's Hospital, Hannover Medical School, [2]Mosaiques Diagnostics and Therapeutics AG, Hannover, [3]Department of Paediatric Nephrology, Hamburg University Hospital, Hamburg, Germany, [4]Department of Paediatric Nephrology, University Children's Hospital, Zurich, Switzerland and [5]Department of Clinical Chemistry, Hannover Medical School, Germany

Correspondence and offprint requests to: Jens Drube; E-mail: jens.drube@web.de
* Both authors contributed equally to this work.

Abstract

Background. The renal Fanconi syndrome (FS) is characterized by renal glucosuria, loss of electrolytes, bicarbonate and lactate, generalized hyperaminoaciduria and low-molecular-weight proteinuria. We studied the urinary low-molecular-weight proteome to identify excreted peptides indicative of a pathogenetic mechanism leading to tubular dysfunction.

Methods. We established a urinary proteome pattern using capillary electrophoresis mass spectrometry (CE-MS) of 7 paediatric patients with cystinosis and 6 patients with ifosfamide-induced FS as the study group, and 54 healthy volunteers and 45 patients suffering from other renal diseases such as lupus nephritis ($n = 8$), focal segmental glomerulosclerosis ($n = 27$), minimal change disease ($n = 7$) and membranous glomerulonephritis ($n = 3$) as controls. Consequently, we conducted a blinded study consisting of 11 FS patients and 9 patients with renal disease other than FS. Additionally, we applied this pattern to 294 previously measured samples of patients with different renal diseases. Amino acid sequences of some marker proteins were obtained.

Results. Specificity for detecting FS was 89% and sensitivity was 82%. The marker peptides constituting the proteome pattern are fragments derived from osteopontin, uromodulin and collagen alpha-1.

Conclusions. CE-MS can be used to diagnose FS in paediatric patients and might be a future tool for the non-invasive diagnosis of FS. The reduced amount of the marker proteins osteopontin and uromodulin indicates loss of function of tubular excretion in all patients suffering from FS regardless of the underlying cause. In addition, the six different fragments of the collagen alpha-1 (I) chain were either elevated or reduced in the urine. This indicates a change of proteases in collagen degradation as observed in interstitial fibrosis. These changes were prominent irrespectively of the stages of FS. This indicates fibrosis as an early starting pathogenetic reason for the development of renal insufficiency in FS patients.

Keywords: capillary electrophoresis; DeToni-Debré-Fanconi-syndrome; mass spectrometry; pathomechanism; proteomics

Introduction

The renal Fanconi syndrome (FS) was first published by Lignac in 1924. Guido Fanconi was the first to describe the entity of complete proximal tubular dysfunction [1]. This is characterized by glucosuria with normal serum glucose levels, generalized hyperaminoaciduria, urinary loss of bicarbonate, lactate, phosphate, potassium and low-molecular-weight proteins. These features are highly variable and a substantial proportion of the patients exhibit only some of them. However, the loss of low-molecular-weight proteins is usually conserved. Cardinal symptoms and signs in children with FS are polyuria, metabolic acidosis, rickets and severe growth failure if left untreated. FS is a constellation of laboratory findings displayed by many different inherited diseases [2] or due to a multitude of exogenous agents such as antibiotics, antiviral agents, chemotherapeutics, bisphosphonate, aristolochic acid that is contained in some Chinese herbs [3], valproate [4], immunosuppressive, antiviral and X-ray contrast agents [5,6].

The complex defect of FS lies within the proximal tubules, where electrolytes, organic substances and water are specifically and actively transported via cell membrane-bound transporters and selective endocytosis [7]. The diagnosis of FS uses analysis of urine to detect glucosuria and low-molecular-weight proteinuria and serum analysis as well as clinical examination. Inherited diseases such as cystinosis and Dent's disease can be proven by molecular genetic analyses. Though many different low-molecular-weight proteins excreted in urine by patients suffering from FS are well known, the majority of these are not used for diagnosis. An exception to this are alpha-1-microglobulin, retinol-binding protein and beta-2-microglobulin. Some of these proteins may neither be the

cause nor are they specific to distinct tubular damage as these proteins are freely filtered in the glomerula and not reabsorbed by defect tubular cells. However, there might be proteins in urine of patients suffering from FS that may play an important part in pathogenesis of FS. In most diseases resulting in FS, the exact cause of the decreasing glomerular filtrate rate (GFR) is unknown. The urinary proteome analysis may reveal marker proteins for renal and extra-renal pathogenetic mechanisms as ∼30% of urinary peptides derive from proteins degredated in other organs, whose fragments are filtrated freely [8].

Analysis of proteome patterns in FS has been carried out previously [9]. However, the authors focussed on low-molecular-weight proteinuria as a sign and did not re-evaluate the pattern obtained by the proteome analysis, which has to be done to confirm the findings [10].

Thus, the aim of this study is to establish a proteome pattern of FS using capillary electrophoresis mass spectrometry (CE-MS) and to re-evaluate it by blinded analysis to investigate even the smallest proteins excreted in FS in the hope of finding a clue to understanding tubular dysfunction in these patients. In addition, we will show that FS can be securely detected using CE-MS. We included paediatric patients with nephropathic cystinosis, Dent's disease, idiopathic FS and children and adults with ifosfamide-induced FS.

Materials and methods

Patients

To establish the urinary proteome pattern of FS, we examined urine samples of seven children with cystinosis (four girls and three boys, median age = 4.3 years, range 2.6–13.3 years) treated at Hannover Medical School and six adult patients with ifosfamide-induced FS treated elsewhere [9], all of whom displayed complete FS. These data were compared with data from 54 healthy volunteers (median age = 14.5 years, range 2.0–30.0 years) for the biomarker discovery. Additionally, urine samples of CKD patients suffering from other renal diseases such as lupus nephritis (SLE, $n = 8$), focal segmental glomerulosclerosis (FSGS, $n = 27$), minimal change disease (MCD, $n = 7$) and membranous glomerulonephritis (MGN, $n = 3$) were used during machine learning to ensure highest model specificity. In order to allow the support vector machine (SVM) to learn the exact strip line between FS and non-FS samples, the additional CKD samples were used to distinguish patients with diminished renal function due to non-FS diseases from FS patients. Such a procedure became necessary, since samples from healthy volunteers and samples from FS patients were more apart than those of CKD patients from FS patients. Thus, we avoided the SVM classifying CKD samples as 'FS' class, and forced the SVM to classify CKD as controls.

In order to test the validity of the proteome pattern, 20 different patients from the outpatients' department of Hannover Medical School, University Children's Hospital Hamburg and University Children's Hospital, Zurich, were included in the blinded arm of our study. Eleven of these patients had a long-standing history of FS. FS was confirmed either by genetic analysis (Dent's disease: mutation in CIC-5 gene), typical laboratory findings (cystinosis being diagnosed by high amounts of cystin in leukocytes) or by fulfilling the criteria of clinical and laboratory findings required for FS. All patients showed glucosuria with normal serum glucose levels, generalized hyperaminoaciduria and urinary loss of bicarbonate, lactate, phosphate, potassium and low-molecular-weight proteins. Three children had ifosfamide-induced FS (all male, ages 6.2, 7.5 and 11 years), five suffered from cystinosis (three males and two females, median age 6.3 years, range 4.8–12.8 years), one male had Dent's disease (age 10.3 years), two patients had idiopathic FS, and in these, all other causes leading to FS were excluded (female, aged 24.2 years, and male, aged 9.4 years). Following KDOQI criteria of the FS patients, 6% had chronic kidney disease (CKD) grade 1, 38% had CKD grade 2, 44% had CKD grade 3, 12% had CKD grade 4 and none had CKD grade 5.

In addition, nine patients with other renal diseases than FS were included for blinded validation. Eight of these children had received a renal transplant (five males, three females, median age 12.8 years, range 8.2–18.7 years), one patient had minimal change disease in remission while on cyclosporine maintenance therapy (female, aged 11.8 years). In order to assess the specificity of the FS-specific urinary proteome pattern, it was additionally applied to samples of 294 patients suffering from various renal diseases: MCD ($n = 18$) [11], MGN ($n = 28$) [11], SLE ($n = 12$) [11], macroalbuminuria ($n = 34$) [12], microalbuminuria ($n = 39$) [12], normalbuminuria ($n = 40$) [12], IgA nephropathy ($n = 42$) [13], renal transplantation ($n = 23$) and renal vasculitis ($n = 58$). Table 1 summarizes the patients' characteristics.

The study was performed with patients' informed consent and the approval of the internal Ethics Review Committee.

Sample preparation

All samples for CE-MS analysis were from spontaneously voided urine and stored at −20°C until analysis. Although urine contains proteolytic enzymes, storage at lower temperatures is unnecessary, as proteins derived from the kidney have already been cleaved at all cleavage sites by the time urine is voided. [14] For proteomic analysis, a 0.7 ml aliquot was thawed immediately before use and diluted with 0.7 ml 2 M urea and 10 mM NH_4OH containing 0.02% SDS. To remove proteins of higher molecular mass, the sample was filtered with Centricon ultracentrifugation filter devices (20 kDa molecular weight cut-off; Millipore, Billerica, MA, USA) at 3000 *g* until 1.1 ml of filtrate was obtained. The filtrate was then applied to a PD-10 desalting column (Amersham Bioscience, Sweden) equilibrated in 0.01% NH_4OH in HPLC grade water to remove urea, electrolytes and salts. We found no significant reduction of peptides and proteins <14 kDa and >90% reduction of albumin (in general >99% reduction). Consequently, we only evaluated peptides and proteins <15 kDa. Finally, all samples were lyophilized, stored at 4°C and resuspended in HPLC grade water shortly before CE-MS analysis, as described [15]. The resuspension volume was adjusted to 0.8 μg/μl, according to the peptide content of the sample as measured by the BCA assay (Interchim, Montlucon, France).

CE-MS analysis

CE-MS analysis was performed with a P/ACE MDQ capillary electrophoresis system (Beckman Coulter, USA) coupled online to a MicroTOF MS (Bruker Daltonic, Germany) [15]. The ESI sprayer (Agilent Technologies, USA) was grounded, and the ion spray interface potential was set between −4.0 and −4.5 kV. Data acquisition and MS acquisition methods were automatically controlled by the CE via contact-close relays. Spectra were accumulated every 3 s, over a range of m/z 350—3000. The average recovery of the sample preparation procedure was ∼85% with a detection limit of ∼1 fmol [15]. The monoisotopic mass signals could be resolved for $z \leq 6$. The mass accuracy of the CE-TOF-MS method was determined to be <25 ppm for monoisotopic resolution and <100 ppm for unresolved peaks ($z > 6$). The precision of the analytical method was determined by assessing (a) the reproducibility achieved for repeated measurement of the same aliquot and (b) by the reproducibility achieved for repeated preparation and measurement of the same urine sample. The 200 most abundant peptides ('internal standard' peptides) were detected with a rate of 98%. The performance of the analytical system over time was assessed with consecutive measurements of the same aliquot over a period of 24 h. No significant loss of peptides and proteins was observed implying the stability of the CE-MS set-up, the post-preparative stability of the urine samples at 4°C and their resistance to oxidizing processes or precipitation [15,16].

Data were accepted only if the following quality control criteria were met: a minimum of 950 peptides/proteins (mean number of peptides/proteins minus one standard deviation) must be detected with a minimal MS resolution of 8000 (required resolution of peaks with $z = 6$) in a minimal migration time interval (the time span, in which separated peptides can be detected) of 10 min. After calibration, the deviation of migration time must be <0.35 min.

Table 1. Patients' characteristics

	FS (data on six adult patients see reference 9)	Training set: healthy controls	Training set: other other renal disease than FS	Blinded control (children with other renal disease than FS)	Blinded control (adults with other renal disease than FS)
n	24	54	45	9	294
Median age (range)	9.5 (2.6–32.9)	14 (2.2–30)	46 (18–69)	11.4 (7.7–18.2)	59 (21–89)
Major diagnosis	Cystinosis: 12 Ifosphamide toxicity: 9 Dent disease: 1 Idiopathic FS: 2	No known disease	SLE:8 FSGS: 27 MCD: 7 MGN: 3	RTX: 8 MCNS in remission: 1	MCD: 18 MGN: 28 SLE: 12 Macroalbuminuria: 34* Microalbuminuria: 39* Normalbuminuria: 40* IgA-nephropathy: 42 RTx: 23 Renal vasculitis: 58
No CKD (KDOQI)	0	54	0	0	63
CKD grade of I (KDOQI)	1	0	2	1	73
CKD grade of II (KDOQI)	8	0	24	3	84
CKD grade of III (KDOQI)	6	0	16	5	57
CKD grade of IV (KDOQI)	1	0	3	0	17
CKD grade of V (KDOQI)	1	0	0	0	0
S-creatinine (μmol/L) ± SD	120 ± 133		132 ± 69	78 ± 25	143 ± 68
Proteinuria (g/day) ± SD	0.64 ± 0.46		1.40 ± 1.82	0.13 ± 0,09	2.56 ± 1.45
Proteinuria (g/day) median and (range)	0.56 (0.16–1.34)		1.46 (0.12–4.13)	0.1 (0.05–0.32)	2.76 (0.08–4.87)

*: in patients with diabetes, SLE, systemic lupus erythematosus; FSGS, focal segmental glomerulosclerosis; MCD, minimal change disease; MGN, membranous glomerulonephritis; RTx, receiver of renal transplant.

Data processing

Mass spectral ion peaks representing identical molecules at different charge states were deconvoluted into single masses using the MosaiquesVisu software [17] (www.proteomiques.com). Only signals observed in a minimum of three consecutive spectra with a signal-to-noise ratio of at least four were considered. MosaiquesVisu employs a probabilistic clustering algorithm and uses both isotopic distribution and conjugated masses for charge-state determination of peptides/proteins. The resulting peak list characterizes each protein/peptide by its molecular mass and normalized migration time. TOF-MS data were calibrated utilizing FT-ICR-MS data as reference masses applying linear regression. Both CE-migration time and ion signal intensity (amplitude) showed high variability, mostly due to different amounts of salt and peptides in the sample. Consequently, CE-migration time and ion signal intensity were normalized based on reference signals by 200 abundant 'housekeeping' peptides generally present in urine, which serve as internal standards [15,18,19]. These 'internal standards' were present in at least 90% of all urine samples with a relative standard deviation of $<100\%$. For calibration, a weighted regression was performed. The resulting peak list characterizes each protein and peptide by its molecular mass [Da], normalized CE migration time [min] and normalized signal intensity. All detected peptides were deposited, matched and annotated in a Microsoft SQL database, allowing further analysis and comparison of multiple samples (patient groups). Proteins and peptides within different samples were considered identical, if the mass deviation was <50 ppm for small peptides or 75 ppm for larger peptides and proteins. The CE migration time deviation was linearly increased over the entire electropherogram from 2 to 5%. These clustering parameters showed minimal error rates and considered increased peak widths at higher migration times. Disease-specific protein/peptide patterns were generated using SVM-based (SVM) MosaCluster software [20]. SVM view a data point (proband's urine sample) as a p-dimensional vector (p numbers of protein used in the pattern), and they attempt to separate them with a $p-1$ dimensional hyperplane. Maximum separation (margin) between the two classes is of additional interest, and therefore, the hyperplane with the maximal distance from the hyperplane to the nearest data point is selected. That is to say that the nearest distance between a point in one separated hyperplane and a point in the other separated hyperplane is maximized. Therefore, all marker proteins are used without any weighting to build up the n-dimensional classification space and to display the dataset in the classification space. Classification itself is performed by determining the Euclidian distance of the dataset to the $n-1$ dimensional maximal margin hyperplane (absolute value of the normal vector) and the direction of the vector (class 1 or class 2).

Statistical analysis

Estimates of sensitivity and specificity were calculated based on tabulating the number of correctly classified samples. Confidence intervals (95% CI) were based on exact binomial calculations performed with MedCalc version 8.1.1.0 (MedCalc Software, Belgium, www.medcalc.be). The ROC plot was evaluated, as it provides a single measure of an overall accuracy that is not dependent upon a particular threshold [21].

The reported P-values were calculated using the natural logarithm transformed intensities and the Gaussian approximation to the t-distribution. Bonferroni adjustments were obtained by applying the standard Bonferroni criterion to the subset of markers that passed the frequency threshold of 70%. The maxT P-values were calculated using the Westfall and Young maxT-procedure [21]. This function computes permutation-based step-down adjusted P-values. A total of 100000 permutations were performed. To ensure stability of the results, we verified that the P-values found by the minP procedure of Westfall and Young were of similar magnitude [22]. Both procedures were implemented as macros in the commercial statistical package SAS (www.sas.com) and were also part of the multi-test R-package of Dudoit *et al*. (see e.g. [23] and references therein), available at www.bioconductor.org.

Sequencing of peptides

Candidate biomarkers and other native peptides from urine were sequenced using CE- or LC-MS/MS analysis as recently described in detail [24].

In addition, MS/MS experiments were performed on an Ultimate 3000 nanoflow system (Dionex/LC Packings, USA) connected to an LTQ Orbitrap hybrid mass spectrometer (Thermo Fisher Scientific, Germany) equipped with a nanoelectrospray ion source. The mass spectrometer was operated in the data-dependent mode to automatically switch between MS and MS/MS acquisition. Survey full-scan MS spectra (from m/z 300 to 2000) were acquired in the orbitrap. Ions were sequentially isolated for fragmentation in the linear ion trap using collision-induced dissociation. General mass spectrometric conditions were electrospray voltage, 1.6 kV; no sheath and auxiliary gas flow; ion transfer tube temperature, 225°C; collision gas pressure, 1.3 mT and normalized collision energy, 32% for MS–MS. The ion selection threshold was 500 counts for MS/MS.

Samples were also analysed using electron transfer dissociation (ETD) [25–27]. Peptides were separated by nRP-HPLC (Agilent 1100; flow split by tee to ∼60 nl/min) and introduced into an ETD-capable Finnigan LTQ quadrupole linear ion trap via nESI, using previously described instrumental parameters [28].

All resulting MS/MS data were submitted to MASCOT (www.matrixscience.com) for a search against human entries in the MDSB Protein Database. The accepted parent ion mass deviation was 50 ppm; the accepted fragment ion mass deviation was 500 ppm. Only search results with a MASCOT peptide score of 20 or better, which also met ion coverage stipulations as related to the main spectral features, were included. Data files from experiments performed on the ETD-enabled LTQ were searched against the NCBI human non-redundant database using the open mass spectrometry search algorithm (OMSSA), with an e-value cut-off of 0.01. The number of basic and neutral polar amino acids of the peptide sequences was utilized to correlate peptide-sequencing data with CE-MS data, as described earlier [24].

Proteinuria expert system AAA

In addition to the measure of the urinary proteome pattern, we used the proteinuria expert system AAA [29] on the 20 patients used for validating the proteome pattern. This expert system uses urinary creatinine, albumin and alpha-1-microglobuline. It is able to distinguish between healthy subjects and patients with pathological proteinuria, as well as between patients suffering from glomerular or tubular proteinuria [29]. This expert system was applied to the patients to double check the results of the proteome analysis by a test used in routine diagnosis by us.

Results

Results of AAA urinary protein expert system

The AAA urinary protein expert system diagnosed all patients with FS as having tubular proteinuria. Of nine controls used in the blinded analysis, six were diagnosed as having no proteinuria. One patient was diagnosed with a mild glomerular proteinuria; this patient had chronic allograft nephropathy 1.5 years after renal transplantation. Two patients were diagnosed as having tubular proteinuria: one suffered from obstruction in the allograft ureter; the other patient showed chronic calcineurin-inhibitor toxicity on allograft biopsy.

Urinary proteome pattern of FS

In order to define FS-specific biomarkers, profiled urine samples of 13 patients suffering from FS were compared with 54 urine samples of healthy volunteers without any history of renal disease. Twenty-four proteins and polypeptides differed significantly in comparison to controls (Table 2). Their sequences were obtained by tandem mass spectrometry. This was successful in 9 of 24 peptides. The pattern of these proteins (Figure 1) was used in SVM learning procedures to establish an FS-specific classification model. In addition to samples of FS patients and controls, 45 datasets of chronic renal diseases were included in the training set to ensure highest classification specificity.

Table 2. Fanconi syndrome-specific biomarkers

Peptide ID	Mass (Da)	CE time (min)	Median FS (counts)	Median HC (counts)	maxT (*P*-value)	Bonferroni (*P*-value)	AUC	Sequence	Protein	Assession No
11 413	981.59	24.8	94	116	0.008	0.0013	0.89	VLNLGPITR	Uromodulin	gi137116
22 625	1169.57	23.7	0	123	0.001	<0.0001	0.86			
23 518	1182.55	28.3	0	220	0.001	<0.0001	0.91			
30 699	1299.58	22.4	303	331	0.001	<0.0001	0.90			
36 769	1405.64	20.1	92	509	0.002	<0.0001	0.90	DGPpGRDGQpGHKG	Collagen alpha-2 (I)	gi124056488
44 802	1526.69	23.9	152	312	0.001	<0.0001	0.91			
46 091	1554.66	28.6	73	76	0.002	<0.0001	0.84			
47 855	1576.74	19.5	186	861	0.001	<0.0001	0.88	YKRKANDESNEHS	Osteopontin	gi129260
49 122	1592.73	19.5	82	432	0.023	<0.0001	0.83			
55 143	1692.80	30.9	438	2498	0.041	0.0209	0.92	PpGEAGKpGEQGVPGDLG	Collagen alpha-1 (I)	gi124056487
56 493	1716.66	20.2	269	450	0.002	<0.0001	0.86			
58 880	1764.68	19.9	148	343	0.002	<0.0001	0.84			
60 355	1798.76	30.3	951	125	0.012	0.0009	0.91	GEpGSpGENGApGQMGPRG	Collagen alpha-1 (I)	gi124056487
61 984	1835.71	19.9	352	1414	0.001	<0.0001	0.92			
67 217	1933.88	21.6	339	516	0.027	0.0144	0.89	GDDGEAGKPGRpGERGPpGP	Collagen alpha-1 (I)	gi124056487
67 263	1934.79	19.9	541	892	0.012	0.0015	0.88			
67 911	1949.89	21.7	0	220	0.001	<0.0001	0.86	GDDGEAGkPGRpGERGPpGP	Collagen alpha-1 (I)	gi124056487
69 979	1996.79	21.0	224	1208	0.001	<0.0001	0.93			
73 434	2067.82	20.6	1089	2120	0.031	0.0111	0.91			
76 839	2128.98	27.0	179	158	0.044	0.0072	0.83	DGKTGpPGPAGQDGRPGPpGppG	Collagen alpha-1 (I)	gi124056487
99 919	2587.20	21.1	122	1036	0.045	0.0162	0.88			
100 020	2589.06	22.6	93	193	0.040	0.0028	0.83			
124 886	3193.38	22.6	2305	1173	0.013	<0.0001	0.83	PpGESGREGAPGAEGSpGRDGSpGAKGDRGETGP	Collagen alpha-1 (I)	gi124056487
170 858	4960.42	20.6	60383	469	0.040	0.0029	0.88			

ID, polypeptide identifier annotated by the SQL database (ID) as described in the methods section; Mass, molecular weight (Da); CE-time, migration time (min) normalized to an array of 200 polypeptides frequently found in urine [21]; median, median of normalized signal intensities, p: hydroxyproline; k: hydroxylysine; the table displays the 24 FS-specific polypeptides defined in our study cohort including protein identification tag, analytical and statistical parameters, fragments' polypeptide sequences and the proteins they are derived from. The obtained MS/MS data were either submitted to MASCOT (www.matrixscience.com) or to OMSSA (http://pubchem.ncbi.nlm.nih.gov/omssa/) for a search against human entries. Accepted parent ion mass deviation was 50 ppm and accepted fragment ion mass deviation was 500 ppm.

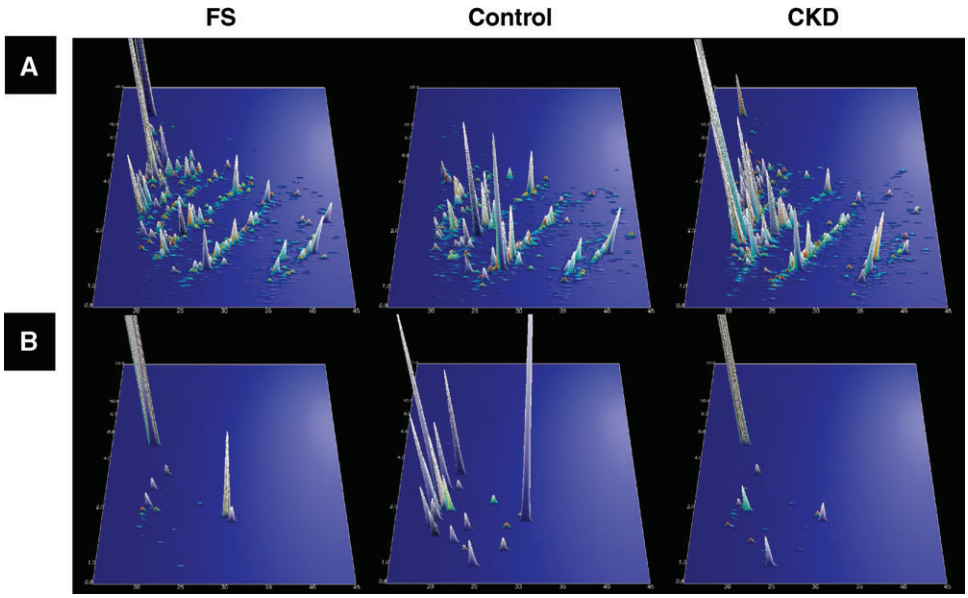

Fig. 1. (**A**) Urinary profiles obtained for patients with FS, age-matched healthy controls and patients with chronic kidney diseases. (**B**) The lower panel depicts the 24 indicative polypeptides defining the FS-specific polypeptide pattern distinguishing patients with FS from healthy controls and chronic kidney diseases (Table 2). Normalized molecular weight is plotted against normalized migration time. The mean signal intensity is given in 3D depiction.

The model allowed discrimination between patients and controls in the training set with a sensitivity of 100% [95% CI 75–100%] and a specificity of 82% [95% CI 73–89%].

In order to validate the defined biomarkers, the model was applied to a set of blinded samples consisting of 11 FS and 9 controls according to the proposed guidelines for clinical proteomics [10]. Ten samples scored positive and 10 negative for FS. After unblinding, 9/11 cases [sensitivity of 82% (95% CI 48–97%)] and 8/9 controls [specificity of 89% (95% CI 52–98%)] were classified correctly. In addition, the model was applied to a test set of 294 samples of various chronic renal diseases. Seventy-three samples scored positive and 221 negative (overall specificity 75%, Figure 2). Table 3 summarizes the obtained classification results.

Discussion

The AAA urinary protein expert system diagnosed all patients with FS as having tubular proteinuria. Of nine controls used in the blinded analysis, six were diagnosed as having no proteinuria. One patient was diagnosed with a mild glomerular proteinuria; this patient had chronic allograft nephropathy 1.5 years after renal transplantation. Two patients were diagnosed as having tubular proteinuria: one suffered from obstruction in the allograft ureter; the other patient showed chronic calcineurin-inhibitor toxicity on allograft biopsy. Thus, the diagnosis of tubular proteinuria is correct in both patients. This indicates that AAA urinary protein expert system was unable to distinguish between FS and other causes of low-molecular-weight proteinuria. In contrast, proteome analysis of spot urine samples using CE-MS can be used to securely diagnose FS. We suggest that the proteome pattern obtained by CE-MS is not merely a surrogate for tubular proteinuria but a specific means of diagnosing FS.

The study focussed on peptides with molecular weight (MW) <15 kDa as larger excreted proteins are well known and intensively investigated. The urinary proteome pattern obtained by CE-MS was specific for FS distinguishing between patients suffering from FS and other causes of low-molecular-weight proteinuria, e.g. both patients of the control group having a tubular proteinuria did not show the FS proteome pattern. A large-scale analysis on 294 samples of patients with renal disease measured previously by CE-MS shows a specificity of 75%. False positive diagnosis was most frequent in patients displaying a gross albuminuria. This may be due to albumin absorbing marker peptides, thus leading to reduced measured amounts of these. As the proteome pattern consists mainly of marker proteins that are found reduced in the spot urine, the absorption capacity of albumin may simulate the FS proteome pattern.

Our study did not distinguish between different diseases causing FS, as the number of samples for most subgroups was too small. However, Vilasi *et al*. [9] reported that Lowe and Dent's disease showed indistinguishable proteome patterns, while idiopathic FS and ifosfamide-induced FS differed from them. Blinded analysis to re-evaluate the pattern obtained by initial analysis was not conducted. This procedure was reported to be mandatory to validate any proteome pattern [10]. Furthermore, two dimensional gel electrophoresis (2DE) and subsequent mass fingerprinting as used by Vilasi *et al*. [9] may be useful in research; however, this method is difficult to use in routine diagnosis. Future examinations will reveal whether or not different causes

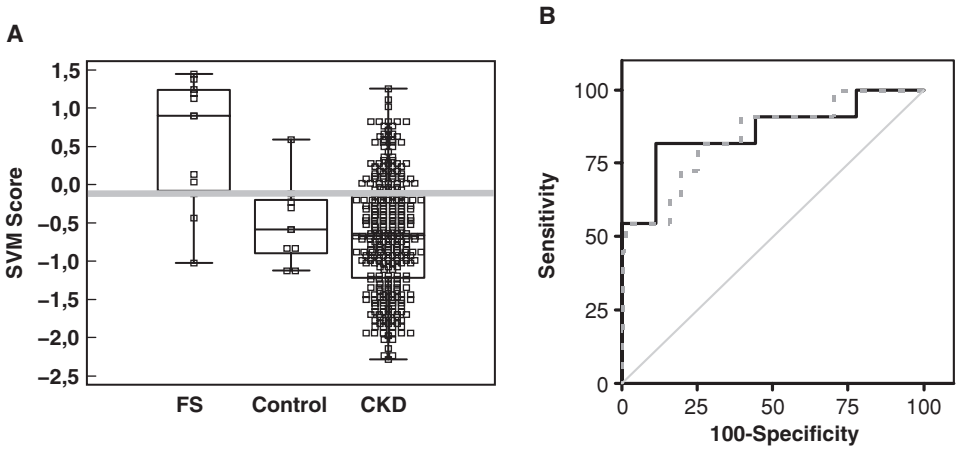

Fig. 2. (**A**) Box-and-whisker plots of SVM score (output of the machine learning algorithm) obtained for the classification of the blinded set and the test set (Table 3). The boxes depict the quartiles Q_1 and Q_3 of each distribution; the statistical medians are shown as horizontal lines in the boxes. The whiskers indicate 3/2 times the interquartile range of Q_1 and Q_3. (**B**) ROC curves of the proteomics diagnosis: using the FS-specific polypeptide panel from Table 2, the SVM score is used as variable in ROC analysis in the 20 samples of the blinded set (11 FS and 9 controls in Table 3, bold line, AUC = 0.86) and in the 294 samples of the test set (AUC = 0.84).

Table 3. Classification results

Clinical condition	Total samples (*N*)	Falsely predicted result (*N*)	Correctly predicted result (*N*)	Correct classification (95% CI)
Training set	112			
FS	13	0	13	100 (75–100)
HC	54	0	54	100 (93–100)
CKD	45	13	32	71 (56–84)
Test set	294			
Macroalbuminuria	34	16	18	53 (35–70)
Microalbuminuria	39	13	26	67 (50–81)
Normalbuminuria	40	4	36	90 (76–97)
IgAN	42	7	35	83 (67–93)
MCD	18	0	18	100 (89–100)
MGN	28	8	20	71 (51–87)
SLE	12	4	8	67 (35–90)
Tx	23	6	17	74 (52–90)
Vasculitis, acute	15	8	7	47 (25–90)
Vasculitis, treated	43	7	36	84 (69–93)
Blinded set	20			
FS	11	2	9	82 (48–97)
Control	9	1	8	89 (52–98)
Total	426			

FS, Fanconi's syndrome; HC, healthy controls; CKD, chronic kidney disease; IgAN, IgA nephropathy; MCD, minimal change disease; MGN, membranous glomerulonephritis; SLE, systemic lupus erythromatosis; Tx, kidney allograft; CI, confidence interval.
This table depicts the classification results obtained for the FS-specific peptide pattern predicting whether or not probands are afflicted by FS.

of FS can be distinguished by CE-MS analysis. Once established, CE-MS could be used as a procedure of clinical diagnosis to identify different causes of FS as it is fully automated.

Proteins with MW >15 kDa in the urine of patients suffering from FS are well documented [9,30]. However, their presence neither revealed why cells of the proximal tubules undergo apoptosis or transition to fibroblasts, nor did they indicated the reason why FS proceeds to renal failure. The majority of the marker proteins of the proteome pattern specific for FS were found in decreased amounts. CE-MS uses house-keeping peptides for normalization that are excreted in a fixed amount per time [19]. Then each peptide concentration is put in relation to housekeeping proteins to obtain quantitative data. Therefore, the observed changes in the proteome pattern are neither due to an elevated amount of other proteins nor due to dilution of urine. Decreased values of marker proteins therefore directly relate to decreased production in the kidney.

Osteopontin derives from tubular cells [31] and is known to be excreted when cells of the tubules are exposed to stress [32]. The function of osteopontin is to remodel the extracellular matrix and inhibition of apoptosis [33] as well as being a protective factor against nephrolcalcinosis [34]. Uromodulin, also known as Tamm-Horsfall protein, is well known to be excreted by cells of the tubules and plays

a major role in combating urinary-tract infections [35]. Both proteins have been reported to be excreted in reduced amounts in FS previously [9,30]. We conclude from our data that they were found reduced in the urine of FS patients because exocytosis of proteins may be impaired in FS. This is true for cystinosis and Dent's disease but also for other causes of FS. It therefore indicates that disturbance of the endoplasmatic reticulum may be a common pathogenetic pathway in FS independent of the underlying cause of FS.

There are no reports on the findings of fragments of collagen alpha-1 in the urine of FS patients. As some fragments were significantly elevated while others were reduced, we conclude that it may indicate a special pathway of collagen degradation in FS. This may be due to the change of matrix metalloproteases (MMP) of subtype MMP 1 and 13 to MMP 2 and 9 in tubulointerstial fibrosis [36]. Collagen alpha-1 fragments were significantly de-regulated in the urine of all 24 patients with FS. We were unable to correlate the excretion of collagen alpha-1 fragments with the grade of CKD. There is strong evidence in the literature that fibrosis is also the common pathway of renal insufficiency in FS irrespective of the underlying causes [37]. Interestingly, the deregulation of collagen alpha-1 fragments was not only observed in cystinotic patients and CKD grades 3 and 4 but also in those children having CKD grades 2 and 1, indicating that tubular fibrosis starts early in FS and may be the reason for progressive renal failure. However, we do not know the role of collagen alpha-1 fragments yet. Most likely the collagen fragments serve as an indicator of renal damage and are not the cause of progressive renal injury.

The results of this study do not answer the question of when renal fibrosis may start in acquired FS and why it continues after the withdrawal of the causal agent. It is also unclear why fibrosis persists during the entire course of the disease in children with congenital FS. Long-term treatment of our cystinotic patients with cysteamine neither stopped nor reversed the deregulated collagen alpha-1 excretion. Future studies will be needed to address these issues.

The proteome pattern established in this study using CE-MS offers a number of future applications in clinical medicine, e.g. the routine diagnosis of renal comorbidity in children with cytotoxic treatment of malignancies may benefit from proteome analysis. Acquired FS was reported to occur in up to 56.7% during cytotoxic therapy in cancer treatment when using ifosfamid [38] or other cytotoxic agents. Of those paediatric patients treated with ifosfamide, 88% developed transient glucosuria [39], while the percentage of those retaining renal impairment ranged from 1.3 to 27% of treated patients [40,41]. The development of symptoms is slow and FS was usually diagnosed only several months after treatment of cytotoxic therapy [42]. A sufficiently reliable and routine test is therefore needed to detect patients with FS before they suffer from renal insufficiency or secondary illnesses such as renal rickets [42]. Future studies on patients with cytotoxic therapy may reveal if the established proteome pattern using CE-MS can detect the onset of FS earlier than conventional methods and if reversible courses can be differentiated from progressive FS.

Furthermore, urinary proteome analysis using CE-MS may help in identifying different types of congenital and acquired FS. A first attempt to achieve this was made by Vilasi *et al*. [9] indicating two different groups in FS: the first made up by Dent's disease ($n = 7$) and Lowe ($n = 1$) and the second by autosomal dominant FS ($n = 3$) and ifosfamide-induced FS ($n = 2$). The small number of patients and the lack of blinded validation of these results indicate the need for further research in this field. As 25–50 patients would be needed for each subgroup in CE-MS analysis, only multi-centre studies will be able to answer the question of FS-subtype-specific proteome patterns. We would like to invite interested physicians and researchers to participate in such a trial.

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Conflict of interest statement. H.M. is the founder and co-owner of Mosaiques Diagnostics, which developed the CE-MS technology and the MosaiquesVisu software. E.S. is an employee of Mosaiques Diagnostics.

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